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# Effect of the Oesophageal Mesenchyme on the Differentiation of the Digestive-tract Endoderm of the Chick Embryo

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**ABSTRACT**—The endoderm of various regions of the digestive tract of 6-day chick embryos was cultured *in vitro* in recombination with the oesophageal or other digestive-tract mesenchymes of 6-day chick embryos, and the differentiation of the epithelium was examined with attention given to the appearance of the stratified squamous epithelium characteristic of the mature oesophageal epithelium.

The oesophageal endoderm developed stratified squamous epithelium in high frequency in the presence of the oesophageal mesenchyme, but in low frequency when recombined with the other mesenchymes, which suggested the supporting action of the oesophageal mesenchyme on the proper differentiation of the oesophageal endoderm. Among the other endoderms of digestive tract, only the proventricular endoderm developed stratified squamous epithelium, when cultured in recombination with the oesophageal mesenchyme. The squamous cells of this epithelium contained numerous tonofilaments as those of the stratified squamous epithelium of the intact oesophagus. The ability to elicit stratified squamous epithelium in the proventricular endoderm was shown to be confined to the oesophageal mesenchyme. Thus, the oesophageal mesenchyme was likely to induce oesophagus-type differentiation at least in the proventricular endoderm.

## INTRODUCTION

It is well known that the epithelial-mesenchymal interaction is prerequisite for the development of the digestive tract of the Aves [1, 14, 24]. Numerous studies have shown that the mesenchyme of the digestive tract can induce some endoderms to differentiate in a mesenchyme-dependent fashion. The duodenal mesenchyme of the young chick embryo was reported to elicit, in the gizzard endoderm associated to it, the intestine-like simple columnar epithelium forming villus- or previllous ridge-like structures, which developed brush-border structure and its enzymes [2, 5–7]. The proventricular mesenchyme induced the associated gizzard or oesophageal endoderm to form compound gland and to produce embryonic pepsinogen or pepsinogen mRNA, which was specific to the differentiated proventricular epithelium [3, 18–20]. Recently, it was demonstrated that the gizzard mesenchyme could induce the duodenal endoderm to become an epithelium forming tubular glands with prominent mucus production, which resembled the intact gizzard epithelium [12], though it is not yet proved whether the chemical nature of the mucus produced in the recombinates was the same as that of the gizzard mucus. Thus, it may be possible that the mesenchymes of most regions of the digestive tract of the young chick embryo possess the ability to induce the region-specific differentiation at least in some endoderms.

The effect of the oesophageal mesenchyme on the differentiation of the endodermal epithelium was also studied so far. The undifferentiated allantoic endoderm was reported to become pluristratified epithelium showing some resemblance to the embryonic oesophageal epithelium when recombined with the oesophageal mesenchyme of the young avian embryo and cultured *in vitro* or *in vivo* on the chorioallantoic membrane of the chick embryo [21, 22], but it became intesinal epithelium with sucrase as well as cloacal epithelium when cultured long enough to achieve full differentiation in the coelomic cavity of the chick embryo [11]. The oesophageal mesenchyme was also reported

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to elicit the yolk sac endoderm to become pluristratified epithelium [9], but the intestinal epithelium with brush border and its enzyme activities also developed in the same type of recombination [9, 10]. The recombination study using the epithelia and mesenchymes of digestive tract demonstrated the heterotypic differentiation of some endoderms cultured in recombination with the oesophageal mesenchyme [23]. Thus, it is still obscure whether the oesophageal mesenchyme has the inductive ability of mesenchyme-dependent differentiation. The present study intended to examine whether the oesophageal mesenchyme has the region-specific inductive influence or not, by the recombination experiment carried out under *in vitro* culture condition. As for the marker of oesophageal differentiation, appearance of the stratified squamous epithelium was examined, since this epithelium was the characteristics of the fully differentiated oesophageal endoderm and was far from being mistaken for other types of epithelium. Though histological is the marker of oesophageal-type differentiation adopted in this study, the stratified squamous epithelium could be regarded as closely related to the cytodifferentiation of the oesophageal epithelial cells, since it appears only in the terminally-differentiated oesophagus of the chick near and after hatching [4, 8, 15, 16].

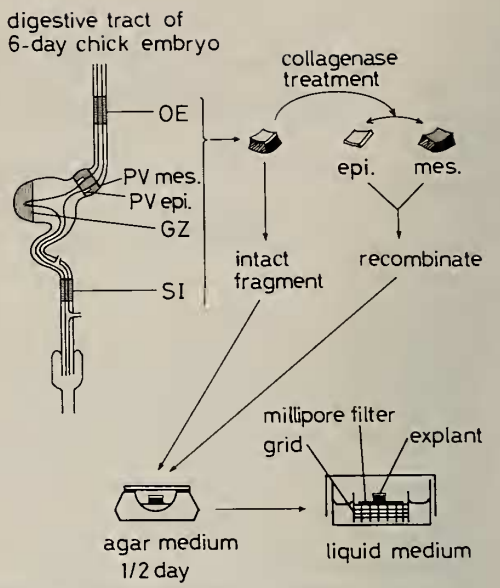



FIG. 1. Diagram showing the mode of combination of epithelia and mesenchymes of the digestive tract. OE, oesophagus; PV, proventriculus; GZ, gizzard; SI, small intestine; epi., epithelium; mes., mesenchyme.

## MATERIALS AND METHODS

### *Animal*

Embryos of the White Leghorn chick were used throughout the experiments.

### *Preparation of tissue fragments*

The middle portion of the oesophagus and of the small intestinal fragment between the bile duct entrance and the yolk stalk, and the apex of gizzard body were removed from 6-day-old chick embryos (Fig. 1), and the endodermal epithelium and mesenchyme were separated with the aid of collagenase (Worthington, Code CLS, 0.03% in Tyrode's solution at 38°C for 1 h). Whole portion of the proventriculus was taken from 6-day chick embryos for the proventricular mesenchyme, while only the posterior part was used for the proventricular endoderm or the intact proventricular fragment, for the purpose of eliminating the possible contamination of the apparent oesophageal tissue (Fig. 1). For the oesophageal mesenchyme of older stages, the fragment just anterior to the crop was taken from 8-day, 10-day and 12-day embryos. The isolated endoderm and mesenchyme were recombined after washing in serum-supplemented (50%) Tyrode's solution and then in Tyrode's solution.

### *In Vitro culture technique*

After cultivation on an agar medium for about half a day at 38°C to ascertain the coherence of endoderm and mesenchyme [23], the recombinates were transfered onto a millipore filter (Nihon Millipore Kogyo K. K., pore size 0.8 $\mu$m). The filter with the recombinate was placed on a stainless-steel grid in a small culture dish containing a liquid medium up to the level of the membrane filter, and the recombinate was cultured at 38°C in 95% air and 5% $CO_2$. The intact fragments of digestive tract were also cultured in the same way.

The culture medium consisted of 75% Medium 199 with Earle's salt (Nissui Seiyaku), 20% 12-day chick digestive organ- and eye-free embryo extract (50% in Tyrode's solution), 5% fetal bovine serum (GIBCO Lab.), and antibiotics (penicillin 100 units/ml, streptomycin 100 $\mu$g/ml). The medium was changed every third day. Since the stratified squamous epithelium was shown in a preliminary experiment to appear in the intact oesophageal explant after 12 days' cultivation in a liquid medium, explants were cultivated for 12 days or longer up to 18 days.

*Histology*

After cultivation, the explants were fixed with ice-cold 95% ethanol for 4 hr. Six $\mu$m paraffin sections were stained with alcian blue (AB)-hematoxylin. Some explants were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C for 2 h and then in 1% $O_sO_4$ in the same buffer at 4°C for 1 h. They were dehydrated with ethanols and embedded in Embedding Resin (TAAB). Ultra-thin sections were stained with methanolic solution of uranyl acetate and with lead citrate.

## RESULTS

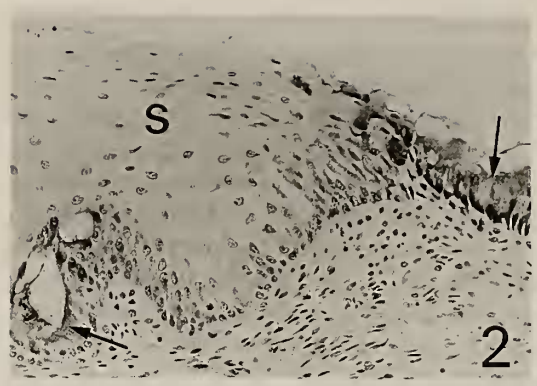
*Appearance of stratified squamous epithelium in the digestive-tract endoderm in the intact digestive-tract fragments and in the recombinates with the oesophageal mesenchyme*

In the explants of intact digestive-tract fragment, the stratified squamous epithelium appeared only in the explants of oesophageal fragment cultured for 12 or 18 days (Table 1, Fig. 2). Columnar epithelium producing AB-stained mucus that might probably correspond to the oesophageal mucous gland epithelium was also found in the oesophageal explants (Fig. 2). In the proventricular explants, columnar epithelium with apical AB-staining differentiated, part of which occasionally developed glandular invagination (Fig. 3). Gizzard explants often formed lots of tubularglandlike structures or intra-epithelial invaginations, which were lined with AB-positive mucus-secretory epithelium. In the small-intestinal explants, simple columnar epithelium with goblet cells appeared. Thus, the appearance of the stratified squamous epithelium was confirmed to be specific to the oesophageal-type differentiation.

When cultured in the presence of the oesophageal mesenchyme, the proventricular endoderm as well as the oesophageal endoderm developed the stratified squamous epithelium after 12 days' cultivation (Table 1). It appeared in the recombinates with the oesophageal endoderm in wide area, while in those with the proventricular endoderm it appeared in various degree, which varied from only a small number of a tiny focus consisting of several squamous cells to a rather wide area (Figs. 4, 5). Transmission electron microscopy showed that numerous tonofilaments were contained in the squamous cells of this stratified squamous epithelium, and ciliated cells were often found (Figs. 6a, b). These were characteristics found in the intact oesophagus during normal development [4, 15] and in the explants of intact oesophagus in the present study (Figs. 7a, b). The possibility that the stratified squamous epithelium found in these recombinates originated from the

Table 1. Appearance of stratified squamous epithelium in the digestive-tract endoderm in the intact digestive-tract fragments and in the recombinates with the oesophageal mesenchyme cultured *in vitro*

| Origin of endoderm | Grafts developing stratified squamous epithelium | |
|---|---|---|
| | Intact fragments | Recombinates |
| Oesophagus | 16/17 (94%) | 7/ 9 (78%) |
| Proventriculus | 0/17 ( 0%) | 20/21 (95%) |
| Gizzard | 0/11 ( 0%) | 0/11 ( 0%) |
| Small intestine | 0/15 ( 0%) | 0/12 ( 0%) |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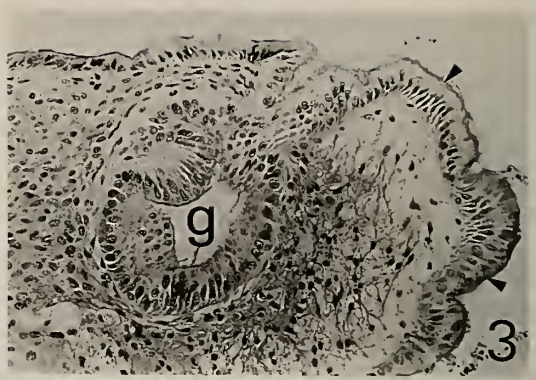
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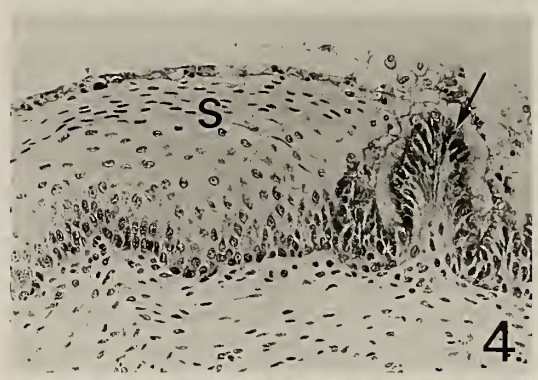
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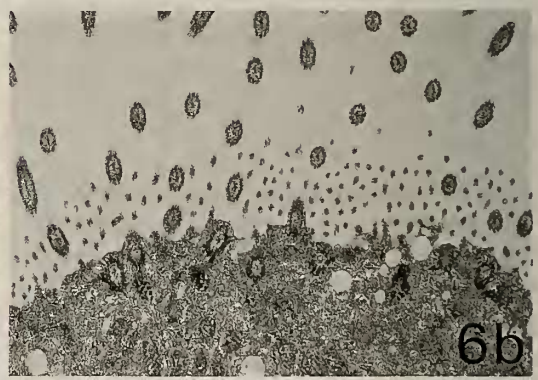
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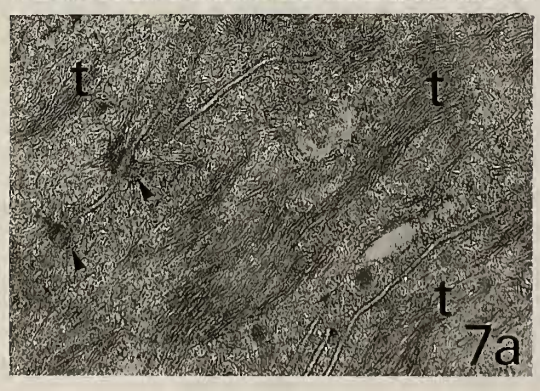
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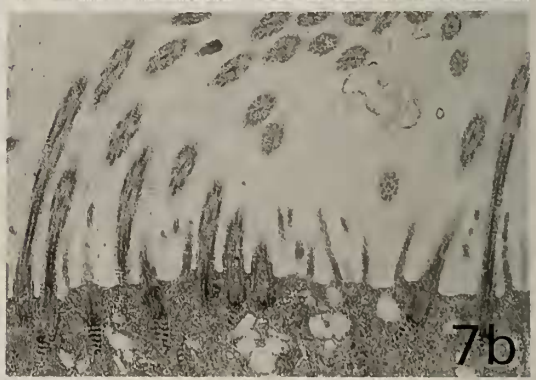
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oesophageal epithelial cells contaminated in the oesophageal mesenchyme was denied by the following findings. Serial sections of the isolated oesophageal mesenchyme revealed the absence of oesophageal epithelial cells, and the cultivation of the mesenchyme alone for 12 days or longer never developed stratified squamous epithelium. In rare cases (1 out of 12), the isolated mesenchyme contained a small epithelial vesicle of unknown origin in the presumptive adventitial tissue outside of the muscular layer, and this epithelial cells did not become stratified squamous epithelium after cultivation. In the other region of the recombinates with the proventricular endoderm and in the recombinates with the gizzard endoderm, mucus-secreting epithelium with abundant undulations or short invaginations appeared (Figs. 4, 5, 8). These epithelial cells may be regarded as the differentiated proventricular or gizzard mucous cells, but discrimination from the oesophageal mucous cells was impossible in the present study. The small-intestinal endoderm became simple columnar epithelium with goblet cells. In a few explants, non-goblet mucous cells were also found.

*Effect of various digestive-tract mesenchymes on the differentiation of the proventricular and oesophageal endoderm*

The proventricular and oesophageal endoderms were found to develop stratified squamous epithelium in the presence of the oesophageal mesenchyme. Then, the effect of other mesenchymes on these endoderms was analyzed.

As shown in Table 2, the proventricular endoderm developed stratified squamous epithelium only in the recombinates with the oesophageal mesenchyme, in which AB-positive mucous epithelium also appeared. The AB-stained epithelium with occasional glandular invagination differentiated in the presence of the proventricular mesenchyme, and the mucus-secretory epithelium forming intra-epithelial invaginations or tubular gland-like structures developed in the presence of the gizzard mesenchyme. In the recombinates with the small-intestinal mesenchyme, AB-positive epithelium was less frequently found.

The oesophageal endoderm developed stratified squamous epithelium in high frequency in the presence of the oesophageal mesenchyme. In the presence of other digestive-tract mesenchymes, stratified squamous epithelium was found in low frequency (Table 2). In the recombinates with the proventricular mesenchyme, AB-positive epithelium with occasional glandular invagination differentiated but even a small focus of stratified squamous epithelium rarely developed (Table 2, Fig. 9). In the recombinates with the gizzard mesenchyme, AB-positive stratified or simple epithelium and stratified squamous epithelium appeared (Fig. 10). In the recombinates with the small-intestinal mesenchyme, AB-positive columnar epithelium appeared. Stratified squamous epithelium was found only in small areas of a few recombinates.

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Fig. 2. An explant of intact oesophageal fragment cultured for 18 days. Stratified squamous epithelium (s) and mucous epithelium (arrows) differentiated. ×230.

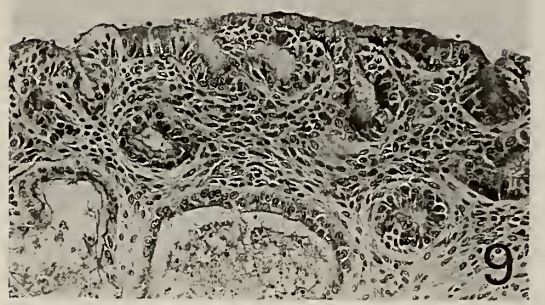
Fig. 3. An explant of intact proventricular fragment cultured for 18 days. A glandular invagination (g) is formed, and the epithelium is often stained with alcian blue in the apical portion (arrowheads). ×230.

Fig. 4. A recombinate of proventricular endoderm and oesophageal mesenchyme cultured for 18 days. Typical stratified squamous epithelium (s) and mucous epithelium (arrow) are seen. ×230.

Fig. 5. Small foci (arrows) of stratified squamous epithelium that appeared in the mucous epithelium found in a recombinate of proventricular endoderm and oesophageal mesenchyme cultured for 18 days. ×230.

Fig. 6. Transmission electron microscopy of an epithelium of a recombinate of proventricular endoderm and oesophageal mesenchyme cultured for 18 days. a. Cells in the middle layer of the stratified squamous epithelium, which are interconnected with each other by desmosomes (arrowheads) and contained abundant tonofilaments (t). n, nucleus. ×24000. b. A ciliated cell. ×12000.

Fig. 7. Transmission electron microscopy of an epithelium of an explant of intact oesophageal fragment cultured for 18 days. a. Cells in the middle layer of the stratified squampous epithelium. Desmosomes (arrowheads) and numerous tonofilaments (t) are seen. ×24000. b. A ciliated cell. ×12000.



*Effect of the oesophageal mesenchyme of aged embryos*

As shown in Table 3, the stratified squamous epithelium appeared only in the presence of the oesophageal mesenchyme of 6-day embryos and not in the presence of the mesenchyme of older embryos, suggesting that the inductive ability of the oesophageal mesenchyme declined rather quickly after 6 days of incubation. The epithelium in the recombinates with the mesenchyme of older embryos became mucus-secreting epithelium with abundant undulations or short invaginations.

TABLE 3. Appearance of stratified squamous epithelium in the 6-day proventricular endoderm cultured in the presence of oesophageal mesenchyme of various developmental stages

| Stage | Grafts developing stratified squamous epithelium |
|---|---|
| 6-day | 20/21 (95%) |
| 8-day | 0/10 ( 0%) |
| 10-day | 0/ 8 ( 0%) |
| 12-day | 0/ 6 ( 0%) |

FIG. 8. A recombinate of gizzard endoderm and oesophageal mesenchyme cultured for 18 days. Mucus-secreting epithelium with abundant undulations or short invaginations appeared. ×230.

FIG. 9. A recombinate of oesophageal endoderm and proventricular mesenchyme cultured for 18 days. Columnar epithelium with apical alcian blue-staining forming glandular invaginations appeared. ×230.

FIG. 10. A recombinate of oesophageal endoderm and gizzard mesenchyme cultured for 18 days. A small area of stratified squamous epithelium (s) is seen. × 230.

TABLE 2. Appearance of stratified squamous epithelium in the proventricular and oesophageal endoderms cultured in the presence of various digestive-tract mesenchymes

| Origin of mesenchyme | Grafts developing stratified squamous epithelium | |
|---|---|---|
| | Proventricular endoderm | Oesophageal endoderm |
| Oesophagus | 20/21 (95%) | 23/26 (88%)[a] |
| Proventriculus | 0/21 ( 0%)[a] | 1/18 ( 6%) |
| Gizzard | 0/11 ( 0%) | 6/11 (55%) |
| Small intestine | 0/12 ( 0%) | 3/11 (27%) |

[a], Explants of the intact fragments as well as the recombinates were included.

## DISCUSSION

The present study clearly showed that the stratified squamous epithelium appeared in high frequency in the proventricular endoderm cultured in recombination with the oesophageal mesenchyme, demonstrating the ability of the oesophageal mesenchyme to induce the differentiation of stratified squamous epithelium. The previous study [23] also reported an occasional appearance of the stratified squamous epithelium in the same type of recombination, but in the present study the stratified squamous epithelium appeared in much higher frequency, which might be due to a better culture condition or a longer cultivation period. The ultrastructural features of the stratified squamous epithelium found in the recombinates were shown to resemble those of the stratified squamous epithelium of intact oesophageal explants, suggesting that both epithelia might represent the same cytodifferentiated state. As shown in the present study, the appearance of stratified squamous epithelium in the proventricular endoderm was confined to the recombinates with the oesophageal mesenchyme. Thus, it was likely that the oesophageal mesenchyme had the region-specific mesenchyme-dependent inducing ability to elicit the oesophagus-like differentiation at least in the proventricular endoderm. The small focus of stratified suamous epithelium would have developed from a single induced cell or a group of a few induced cells in the recombined proventricular endoderm. The inductive ability of the oesophageal mesenchyme to elicit stratified squamous epithelium in the proventricular endoderm was shown to decline rather quickly after 6 days of incubation, while the ability to elicit pluristratified epithelium in the allantoic endoderm was reported to be retained until the 11th days of incubation [21]. This discrepancy might be due to the difference in the responding tissue and/or to the difference in the examined histological marker in the two experiments.

Regional difference in the responsiveness of the digestive-tract epithelium to the inductive stimuli of the mesenchyme was repetitively reported [3, 5, 20, 23]. The responsiveness to the oesophageal mesenchyme also showed regional difference in the digestive-tract endoderms, and was found to be confined to the endoderm of the proventriculus, which situated next to the oesophagus, at least in the 6-day-old chick embryo. That the competence for the inductive action of the mesenchyme was high in the endoderm of its neighboring region(s) was reported also in the case of intestinal differentiation induced by the duodenal mesenchyme [5] or of the induction of pepsinogen or pepsinogen mRNA by the proventricular mesenchyme [3, 20]. As for the high responsiveness of the proventricular endoderm, the following three possibilities may be considered; 1) some of the proventricular endodermal cells might be induced to change their fate and to become oesophageal-type epithelium; 2) undifferentiated cells capable to adopt oesophageal or proventricular fate according to the influence of the mesenchyme might be contained in the proventricular endoderm; 3) a small number of oesophageal cells which are to be lost through some selective mechanism(s) during normal development in the proventriculus might be distributed even in the posterior part of the proventriculus. It needs further extensive analysis to know which is the case.

The present study also showed that the differentiation of the oesophageal endoderm was influenced by the mesenchyme. The appearance of stratified squamous epithelium was low in the oesophageal endoderm cultured in recombination with the mesenchyme other than that of the oesophagus. Thus, the other mesenchyme might provide only an inappropriate condition for the differentiation of the stratified squamous epithelium or exert an inhibitory effect on its differentiation. The proventricular mesenchyme was known to possess an ability to induce proventricular differentiation [3, 20], and this effect may have contributed to rare appearance of the stratified squamous epithelium in the recombinates with the proventricular mesenchyme. On the contrary, the oesophageal mesenchyme provided a suitable condition for the normal differentiation of the oesophageal endoderm. Whether this effect of the oesophageal mesenchyme on the oesophageal endoderm may be a nutritional one or be related to the inductive influence as exerted over the proventricular endoderm is not known. The appearance

of the stratified squamous epithelium even in the recombinates with the mesenchyme other than the oesophageal one, though in a rather low frequency, suggests that the oesophageal endoderm of the 6-day chick embryo has already been determined to some extent, as suggested by the presence of self-differentiation potency in the oesophageal endoderm of this stage [17].

At least at 6 days of incubation and probably at later stages during normal development, the oesophageal mesenchyme would interact mainly with the oesophageal endoderm likely to be endowed with the oesophageal fate. Since the oesophageal endoderm of 6-day embryos has also a potency of heterotypic differentiation into proventricular-type [3, 20], the inductive effect of the oesophageal mesenchyme might play, in concert with its probable supporting action that provides the approprtiate condition(s), an important role in assurring the correct differentiation of the oesophageal endoderm, as suggested by Mizuno (1975) [13].

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